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LIPOSPHERES OF ANTIRETROVIRAL PROTEASE INHIBITORS: AN APPROACH FOR BETTER TREATMENT OF DRUG RESISTANT PARASITES AND HIV-MALARIA CO-INFECTION

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ABSTRACT

Parasite resistance to antimalarial drugs is a life-threatening problem, and novel agents acting on enzymes essential for parasite metabolism, such as proteases, are attractive targets for drug development. Recent studies have shown that antiretroviral protease inhibitors (ARPIs) possess some antimalarial activity by inhibiting an aspartyl protease of malaria parasite. The present study deals with the formulation of lopinavir-ritonavir lipospheres (LOP-RIT LS) to enhance their activity in the treatment of drug resistant malaria as well as a malaria human immunodeficiency virus (HIV) co-infection. LS were prepared by using hot melt homogenization method. Fourteen different formulations (F1-F14) have been prepared by varying different parameters like lipid concentration, surfactant type and surfactant concentration. The prepared formulations were investigated for particle size, surface morphology, entrapment efficiency (EE) and in-vitro drug release. Among all formulations, F9 was found to be optimized, having a particle size (140.13±0.7 NM), EE (82.39±0.41% LOP, 83.91±0.42 RIT) and extended drug release (86.45±0.43% LOP, 83.79.12±0.42% RIT). Further characterization of optimized formulation for differential scanning calorimetry (DSC) confirmed entrapment of both the drugs in LS, X-ray diffraction (XRD) revealed crystalline nature and stability studies prove stability. In case of in-vivo study, LS formulation was found superior to marketed product in its ability to suppress parasite.

KEYWORDS: Parasite resistance, Malaria HIV co-infection, Antiretroviral protease inhibitors, lopinavir, ritonavir, lipospheres.

INTRODUCTION

Malaria is a major cause of morbidity and mortality, estimated to infect about 300 to 500 million and to kill about 1 to 2 million people annually.[1] It is endemic in most tropical and subtropical regions of the world.[2] Many effective antimalarial drugs are available, but development of drug resistance has dramatically reduced their effectiveness in most regions where malaria is endemic. This Parasite resistance to antimalarial drugs is a serious threat to human health.[3,4] Furthermore, HIV/AIDS overlap malaria in wide geographical regions, resulting in co-infection and the interaction between these two diseases clearly has major public health implications.[5] When HIV and malaria parasite are present as a co-infection, they enhance each other's pathogenicity.[6,7] There are examples of harmful effects of drug interaction between antiretroviral therapy and antimalarial therapy for the coinfected patient [8] and alterations in the pharmacokinetics of several drugs that are used to treat HIV and malaria.[9,10] An antiretroviral treatment regimen that not only inhibits HIV replication and improves immunologic competency but also shows an inhibitory activity against the Plasmodium parasite is highly desirable. There is an increasing evidence that ARPIs may feel that role[1,4,11,12] as aspartic proteases have been found to play key roles in the biology of malaria parasites and HIV-1.[11] Plasmodium falciparum, the most virulent human malaria parasite, expresses a number of aspartic proteases, known as plasmepsins[13] and recent studies have shown that HIV protease inhibitors such as LOP, RIT, saquinavir etc can inhibit the in vitro growth of Plasmodium falciparum at or below concentrations found in human plasma after oral drug administration in both drug sensitive and drug resistant parasites.[4,11,12] Aspartic protease inhibitors such as LOP and RIT are BCS IV drugs.[14] The main challenge in LOP/RIT formulation is their poor bioavailability, mainly due to poor aqueous solubility, high first pass metabolism and efflux.[15,16] In addition to this, half life of both drugs is very short which results in increasing pill burden and cost of therapy that has led to a reduction in the number of patients receiving the therapy.

Various lipid based carrier system developed to improve the solubility, bioavailability and target intestinal lymphatic delivery, such as lipid solutions, microemulsions, liposomes, self-emulsifying delivery systems, micellar solutions, and solid lipid nanoparticles.[17-20] LS are novel drug delivery vehicles, water-insoluble lipid spheres forming a solid hydrophobic core,

with a layer of phospholipids embedded on the surface of the core. Drugs or other biologically active agents may be contained in the hydrophobic core, adhered to the phospholipids, or a combination thereof.[21] LS have more advantages over other delivery systems, such as better physical stability, low cost of ingredients, ease of preparation and scale-up, high dispersibility in an aqueous medium, high entrapment of hydrophobic drugs, controlled particle size and extended release of entrapped drug.[22-24]

The main objective of this study was to formulate LS of the ARPIs, LOP and RIT, to enhance their activity in the treatment of drug resistant malaria as well as malaria HIV co-infection. LS were prepared by varying different parameters like lipid concentration, surfactant type and surfactant concentration using hot melt homogenization method & investigated for particle size, shape, entrapment efficiency, in vitro drug release study.

MATERIALS

LOP and RIT were kindly gifted by Mylan Pharmaceuticals, Nashik, India. Stearic acid (SA) and polyvinyl alcohol (PVA) were procured from Loba Chemie, Mumbai. Poloxamer 188 was provided as a gift sample by BASF, Mumbai. Acetonitrile and egg phosphtidylcholine were purchased from Research Lab Fine Chem Industries, Mumbai. All other chemicals and solvents were of analytical grade.

METHODS

Preparation of LOP-RIT LS

Fourteen different formulations (F1-F14) have been prepared using different composition (Table 1) by melt dispersion technique/ hot homogenization method. First of all, lipid phase comprising of the required quantity of SA was melted in a water bath maintained at 70-72 °C. Both the drugs were dispersed into the molten lipid. The aqueous phase was prepared separately by heating a blend of phosphate buffer (pH 6.8), required quantity of surfactant (PVA or Poloxamer 188) and egg phosphatidylcholine to 70-72 °C. The aqueous phase was slowly transferred to lipid phase. The mixture was homogenized for 15m using high speed homogenizer (T-10 basic ultra turrax IKA). The milky dispersion was rapidly cooled down to 20°C by immersing the formulation in an ice bath with continued agitation to yield uniform dispersion of LS. Then obtained LS were isolated by filtration and air dried.

Table 1: Composition of LOP-RIT loaded LS.

Batch Code	Lipid Core (Drugs: SA)	Surfactant (%)		Egg Phosphatidylcholine (%)
		PVA	Poloxamer 188	
F1	1:1	0.1	-	0.5
F2	1:1	0.2	-	0.5
F3	1:1	0.3	-	0.5
F4	1:1	0.4	-	0.5
F5	1:1	-	0.1	0.5
F6	1:1	-	0.2	0.5
F7	1:1	-	0.3	0.5
F8	1:1	-	0.4	0.5
F9	1:2	0.4	-	0.5
F10	1:3	0.4	-	0.5
F11	1:4	0.4	-	0.5
F12	1:2	-	0.4	0.5
F13	1:3	-	0.4	0.5
F14	1:4	-	0.4	0.5

Characterization of LS

EE

The entrapped drug concentration was determined by lysis of the LS**.** An accurately weighed quantity of drug loaded LS (10 mg) was pulverized and dissolved in 10 ml acetonitrile. The solution was subjected to sonication for 15 minutes (Biomedica, BMI-599), filtered and absorbance of the sample solutions at 239nm and 259nm were measured and from the absorbance values, the concentration of drugs in the sample solution was determined by simultaneous equation method.

Particle Size Analysis

The particle size of all LS formulations was determined by light using the laser diffraction particle size analyzer, NANOPHOX (NX0088). Liquid formulations were further diluted with distilled water to make 5% v/v. The particle size distributions were estimated by setting the intensity of the scattered light at a wavelength of 750 nm and the scattering angle (θ) of 90^0.

Photomicroscopic Analysis

A small quantity of LS was suspended in 1ml water and a drop of this suspension was placed on a slide for morphological examination under Motic digital microscope.

Scanning Electron Microscopy (SEM)

The surface morphology of the solid LS was studied by SEM. The samples for SEM were

prepared by lightly sprinkling powder on a double adhesive tape stuck to an aluminum stub which was then placed in the scanning electron microscope (JEOL JSM- 6360, Japan) chamber. The samples were then scanned randomly and photomicrographs were taken.

DSC Analysis

The DSC thermograms were recorded for both the drugs, plain LS and loaded LS using differential scanning calorimeter (TA-60LS WS thermal analyzer, Schimadzu, Japan). Approximately 2-5 mg of each sample was heated in aluminum pan (Al-Crucibles, 40 Al) from 30^0C to 300^0C at a heating rate of 10^0C/min under a stream of nitrogen at a flow rate of 50ml/min.

Powder XRD

X-ray diffraction patterns of the powdered samples of both the drug and formulation were scanned by X-ray diffractometer (Philips, PW3710), from the diffraction angle (2θ) 5 to 50^0. Diffraction pattern for both the drugs and loaded LS were obtained.

In Vitro Drug Release Studies

Amount of LS equivalent to 166 mg of total drug, was filled in a capsule and in vitro drug release was performed in acidic buffer (pH-1.2) for first two hours and in phosphate buffer (pH-6.8) afterwards using USP type I dissolution tester, under stirring at 50 rpm. Samples were withdrawn at regular time interval (every hour) and the same volume was replaced immediately by fresh phosphate buffer. The sample solution was filtered and analyzed for drug content by measuring absorbance at 239nm (RIT) and 259nm (LOP). The amount of drug released was calculated using simultaneous equation. Drug release of optimized batch was compared with the release of plain drugs (LOP and RIT) and marketed formulation.

In Vivo Antimalarial Activity

The in vivo antimalarial activity of ARPIs was determined using the lethal murine malaria model of Plasmodium berghei in Swiss albino mice. Eighteen healthy albino mice (average weight 35g) were selected for the evaluation purpose. They were divided into three groups (each group consisted of 6 rats).

Group A (LS formulation)
Group B (Marketed formulation)
Group C (Vehicle/water)

All the mice were infected intravenously (in the tail vein) with 10^5 parasitized erythrocytes from an infected donor mouse. Mice received vehicle or drug (as per their groups) in a 100µl oral solution twice a day for 8 days, beginning 24h postinfection (p.i.). Parasitemia was monitored by daily microscopic examination of Giemsa-stained blood smear.

Table 2: Description of groups.

Groups	Group A	Group B	Group C
Group description	Mice received optimized LS formulation	Mice received marketed formulation of LOP and RIT (LOPIMUNE)	Vehicle treatment
No. of mice	6	6	6
Dose	50 mg/kg	50 mg/kg	-

Stability Studies

In any rationale design and evaluation of dosage forms of drugs, the stability of the active component must be major criteria in determining their acceptance or rejection. Stability study was carried out according to ICH and WHO guidelines. Optimized formulation was kept at 4^0C, room temperature (RT) and in the humidity chamber, maintained at $40^0 \pm 2^0$C and 75% ± 5% RH for three months. At the end of studies, the sample was analyzed for the entrapment efficiency and particle size.

RESULTS AND DISCUSSION

EE: The EE of different LS formulations is shown in Table 3. LS were found to have good entrapment efficiency with maximum drug loading up to 84.52±0.42% of LOP and 85.19± 0.43% of RIT. The EE was found to be increased with an increase in the lipid concentration as well as the surfactant concentration. LS prepared by using PVA shown higher EE as compared to LS prepared by using poloxamer 188. The possible reason might be a difference in HLB values of poloxamer (HLB=29) and PVA (HLB=18). Higher hydrophilisity of poloxamer might have reduced the solubility of drugs in the lipid.

Particle Size Analysis

The formulations containing PVA showed size distribution in the range of 140nm to 350nm while formulations with poloxamer 188 showed particle size ranging from 80nm to 180nm (Table 3). The average particle size of the selected formulation was found to be 140 nm (Figure 1). The results clearly reveal, when other parameters were kept constant, the particle size was inversely proportional to surfactant concentration. LS formulated by using poloxamer as surfactant found to have lesser particle size as compared to those prepared by

using PVA as surfactant.This may be due to higher hydrophilisity of poloxamer 188. As the lipid concentration increased, particle size also increased. The reason behind this might be increased viscosity of medium which prevent diffusion of the drug. At higher lipid concentration, efficiency of homogenization decreases, resulting in aggregation of particles. The increase in the lipid concentration was found to be accompanied with decrease of the percentage drug released.

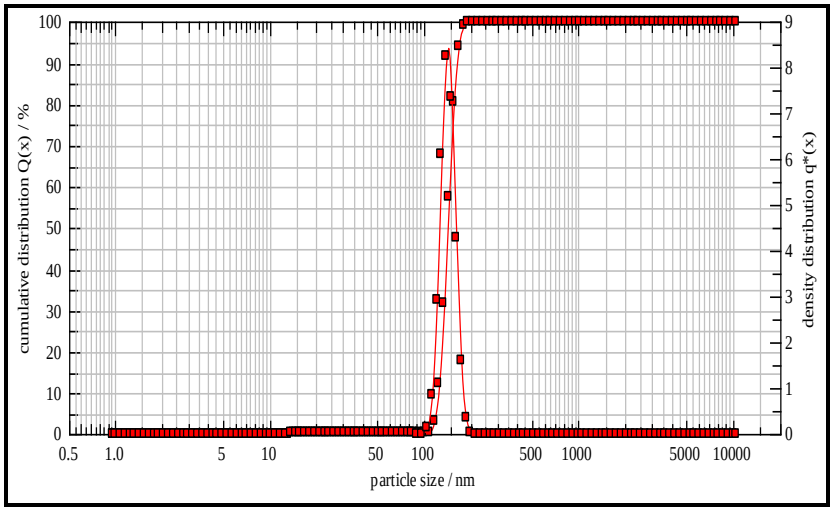

Fig. 1: Average particle size of selected batch (F9)

Table 3: EE and particle size (F1-F14).

Batch code	EE (%)		Particle size (nm)
	LOP	RIT	
F1	65.23±0.33	69.82±0.35	318.5 ± 1.59
F2	69.23±0.35	72.2±0.36	210.55 ± 1.05
F3	73.15±0.37	75.0 ±0.38	196.36 ± 0.98
F4	74.01±0.37	78.2 ±0.39	132.43 ± 0.66
F5	58.45±0.29	59.0 ±0.30	127.55 ± 0.64
F6	61.98±0.31	61.8±0.31	122.61 ± 0.61
F7	64.82±32	64.69±0.32	105.67 ± 0.53
F8	68.55±0.34	67.84±0.34	83.49 ± 0.42
F9	82.39±0.41	83.91±0.42	140.13 ± 0.7
F10	83.46±0.42	84.59± 0.42	175.5 ± 0.88
F11	84.52±0.42	85.19± 0.43	250.5 ± 0.1.25
F12	71.65±0.36	71.42± 0.36	150.55 ± 0.75
F13	73.91±0.37	73.2 ±0.37	165.47 ± 0.83
F14	76.5±0.38	75.1± 0.38	170.57 ± 0.85

Photomicroscopic Analysis

The photomicrograph (Fig.2) of the LS formulation (F9) clearly reveals the uniform spherical form of LS particles showing the solid lipid core and the coat of phospholipid.

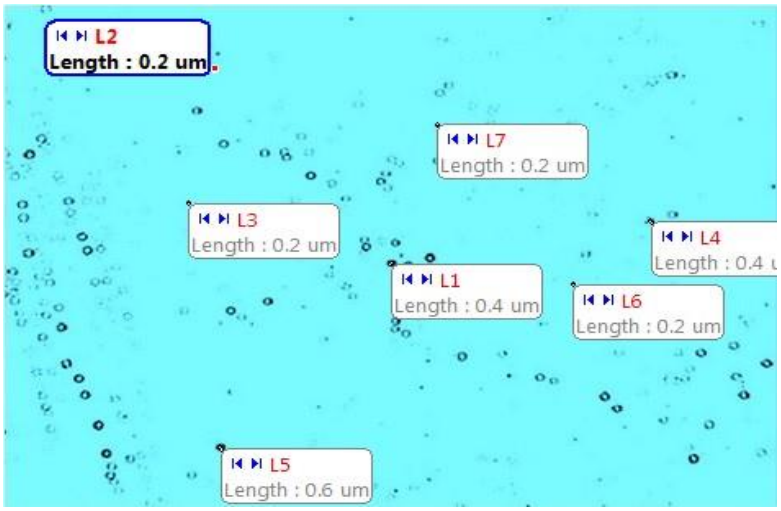

Fig. 2: Photomicrographs LS formulation (F9)

SEM

The surface morphology and shape of the LS were analyzed by scanning electron microscopy (Fig. 3). The LS were found to posses discrete particles with irregular surface. This is usually obtained when phosphatidylcholine is used as a coating.

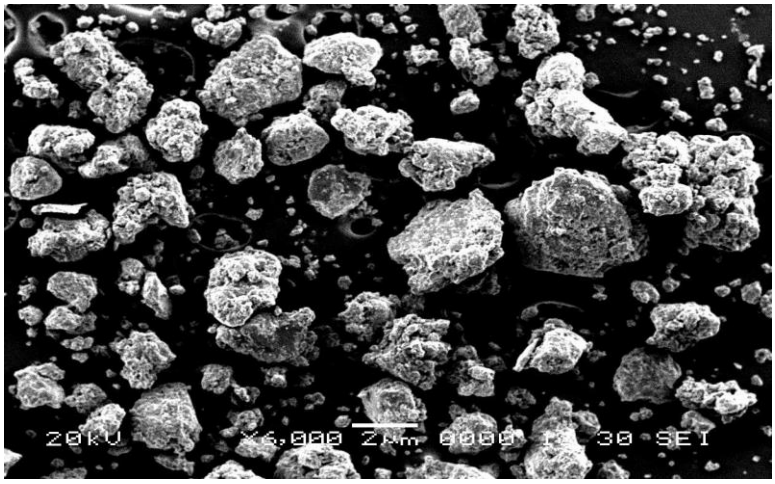

Fig. 3: Scanning electron micrographs LS formulation

DSC Analysis

DSC thermograms (Fig.4) of LOP and RIT showed endothermic peaks at 127.4° and 121°C respectively, which are reported melting points of both the drugs. Plain LS showed an endothermic peak at 57.4°C while drug loaded LS prepared by same method showed sharp endothermic peak at 56.4°C and disappearance of the drug peaks clearly indicating the molecular dispersion of both the drugs into the LS formulation and both drugs exist in amorphous state rather than crystalline state. Reduction in SA melting point and the disappearance of the drug peaks in loaded LS suggests inclusion of both the drugs in the lipid.

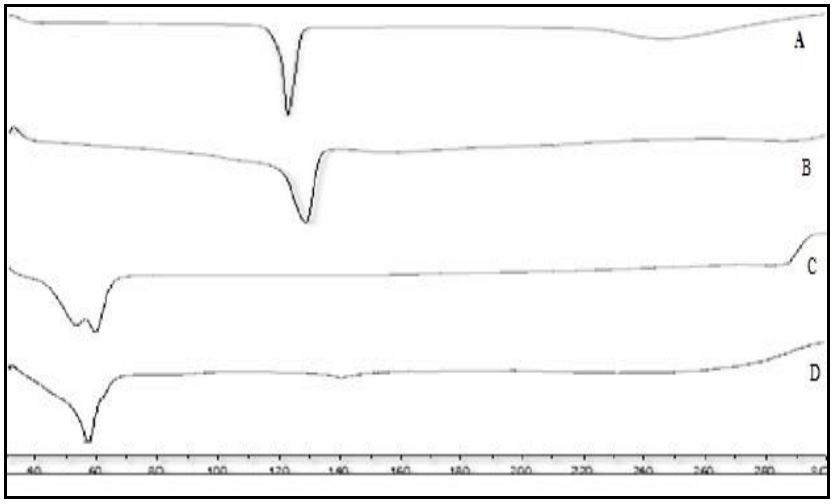

Fig. 4: DSC thermograms of (A) RIT (B) LOP (C) Plain LS (D) Drug loaded LS.

Powder XRD

XRD pattern (Fig. 5) of LOP and RIT had all characteristic peaks, suggesting their crystalline nature. Absence of all intense characteristic peaks of both the drugs LOP-RIT LS indicates presence of LOP and RIT in amorphous form and efficient miscibility of drugs in SA.

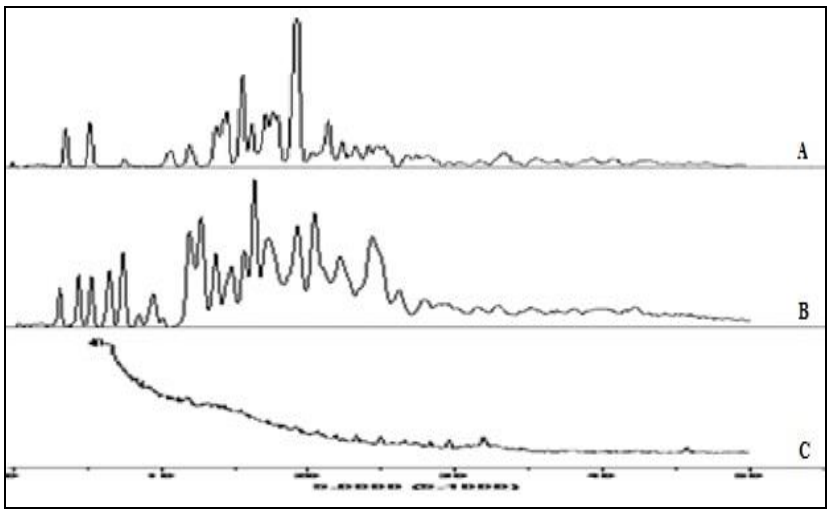

Fig. 5: XRD pattern of (A) LOP (B) RIT (C) LS formulation.

In Vitro Drug Release

Typical drug release profiles of all LS formulations are shown for both the drugs LOP (Fig. 6) and RIT (Fig. 8). All the formulations have shown sustained release for both drugs. The drug release of optimized formulation is compared with the drug release profile of plain drug LOP (Fig. 7) or RIT (Fig. 9) and the marketed formulation containing combination of both drugs. As compared to plain drug and marketed formulation, LS were found to have better sustained release. Sustained release from LS is attributed to a lipid matrix imparting barrier to drug release.

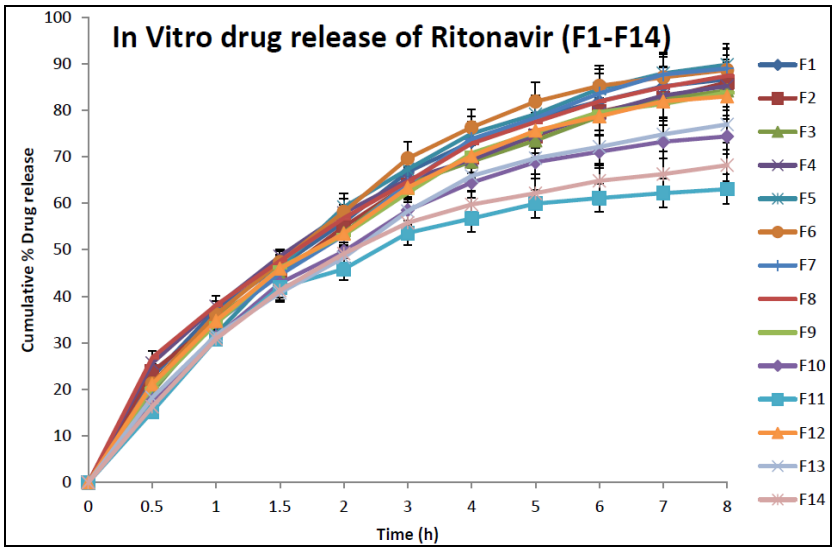

Fig. 6: In vitro release of RIT from all LS formulations (F1-F14)

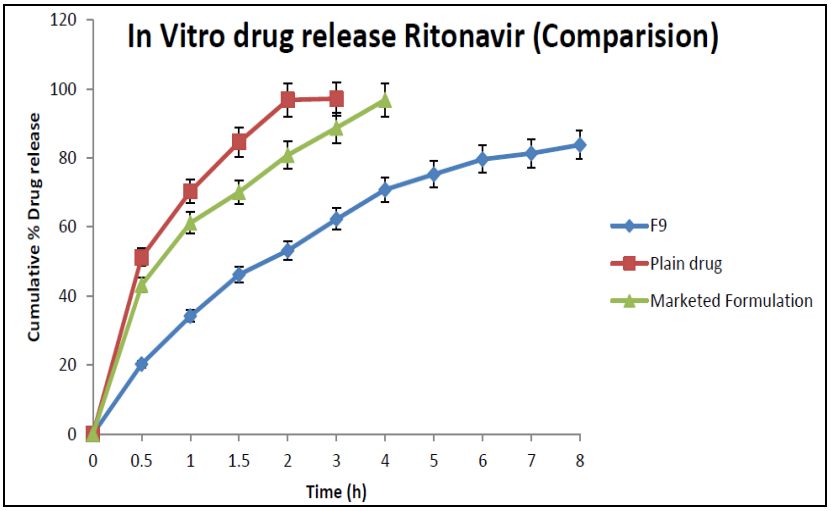

Fig. 7: Drug release of RIT (F9, marketed, plain drug)

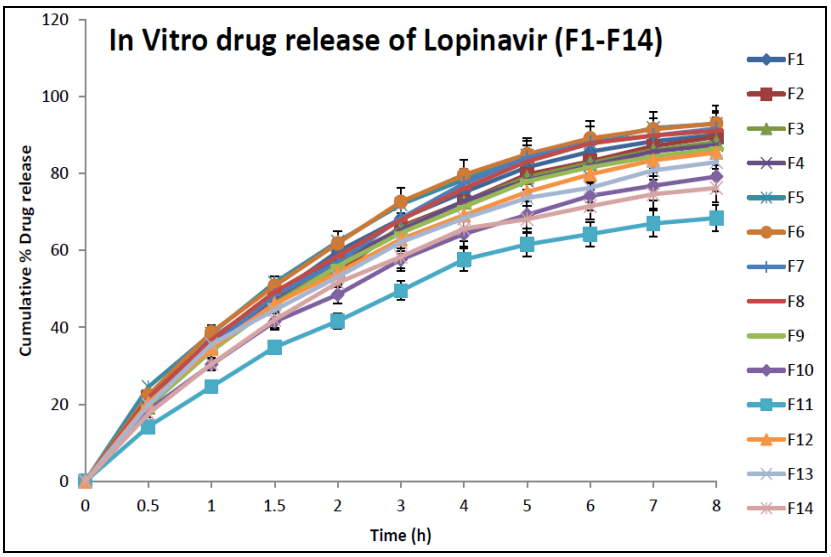

Fig. 8: In vitro release of LOP from all LS formulations (F1-F14)

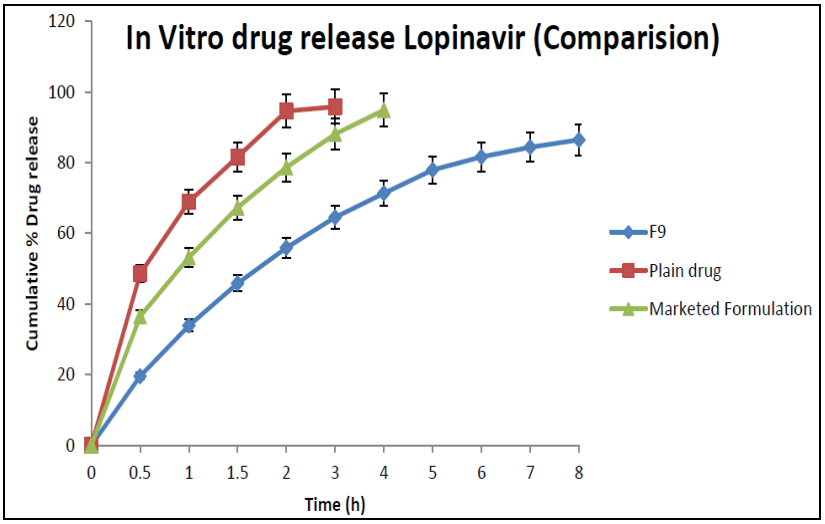

Fig. 9: Drug release of LOP (F9, marketed, plain drug)

In Vivo Antimalarial Activity

In the present study, parasitemia was monitored by Giemsa staining. Malaria parasitemia is a measurement of the amount of Malaria parasites in the patient's blood and an indicator for the degree of infection. Average percent parasitemia of groups A and B was compared with group C (Fig. 10, Fig. 11). After treatment Group A and B both shown significant antimalarial activity against P. burghei. The optimized formulation (F9) shown better antimalarial activity as compared to available marketed formulation of ARPIs (Lopimune).

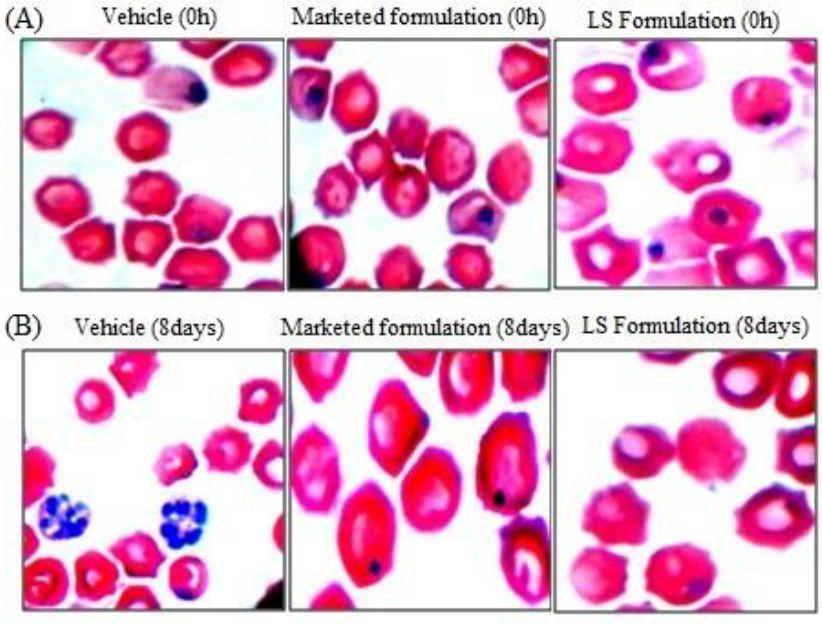

Fig. 10: Photographs showing parasitemia (A) Before treatment (B) After treatment

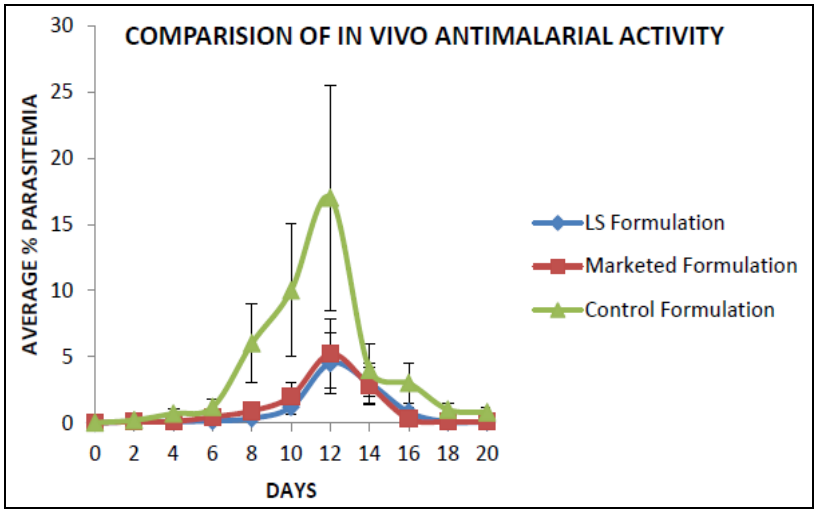

Fig. 11: In vivo efficacy of HIV-1 protease inhibitors against *P. berghei*

Stability Studies

The stability studies were carried out on optimized formulation. Considering the results of stability studies carried out for 3 months, LS formulation was found to be stable in all three storage conditions, but lowest change was observed when stored in refrigerator (Table 4). The particle size of the LS was found to be enhanced after storage for 3 months, but size growth in glyceride particles is common. The particle size of the formulation was enhanced from 140.13 ± 0.7 nm to 154.63 ± 0.75 nm which is minimal.

Table 4: Stability Studies of Optimized Formulation.

Conditions	Entrapment efficiency (%)		Particle size (nm)
	LOP	RIT	
Initial	78.01±0.04	78.2 ±0.11	140.13 ± 0.97
One Month			
5^0C±3^0C	77.18±0.08	77.81±0.008	141.43±0.01
25±2 ◦C/60±5% RH	76.51±0.2	76.21±0.004	142±0.06
40^0C / 75%RH	75.51±0.04	75.45±0.02	145.23±0.01
Two Months			
5^0C±3^0C	76.98±0.1	76.01±0.004	141.98±0.04
25±2 ◦C/60±5% RH	75.01±0.004	75.29±0.04	144.03±0.01
40^0C / 75%RH	73.41±0.19	73.41±0.01	149.73±0.02
Three Months			
5^0C±3^0C	76.01±0.08	75.04±0.01	142.45±0.02
25±2 ◦C/60±5% RH	74.51±0.2	74.19±0.03	146.11±0.04
40^0C / 75%RH	72.91±0.4	72.73±0.02	154.33±0.03

CONCLUSION

The drug loaded LS were successfully prepared by melt dispersion technique and finding of the study suggest that, LS can be considered as a promising carrier system for effective

delivery of ARPIs in treatment of drug resistant malaria as well as HIV- malaria co-infection. LS were able to entrap both the drugs, LOP and RIT, simultaneously to satisfactory levels and sustain their release. Entrapment and drug release were found to be affected by surfactant and lipid concentration, thus are critical parameters to be controlled in formulation. Furthermore, these LS were found to have high stability and superior antimalarial activity as compared to marketed preparation. Hence this particular combination of LOP and RIT in LS form has potential to be developed as a sustained drug delivery system which will improve patient adherence to the therapy as it may reduce the pill burden. In developing countries where malaria is endemic and people suffering from HIV-malaria co-infection, ARPIs LOP and RIT in form of LS can be a good option for therapy.

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